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14. ABSTRACT The purpose of this proposal is to detect the role of Stat3 activation during prostate cancer progression. Amultifaceted approach is being used to accomplish the proposed research goals. Significant progress was made insupport of Task 1 in that we were able to overexpress activated Stat3 (Stat3C) in tow human prostate cell lines. Bothlines were characterized and at least on line (MDAPLa 2b) exhibits molecular alterations consistent with Stat3 activation. In support of Task 2, we have begun characterization of the Pb.Stat3C transgenic line and the preliminaryhistopathological findings indicate that expression of activated Stat 3 may confer a neoplastic phenotype. Theproposed bigenic cross (Pb.Stat3C x BK5.IGF-1) has also been initiated as well as an alternate strategy due to thelimited fertility of the BK5.IGF-1 transgenic line and the apparent compromise in viability of the bigenic offspring. Our results to date have been promising and should further our understanding of the role of Stat3activation in prostate cancer progression.

15. SUBJECT TERMS
STAT3, EMT, BK5, IGF-1, PB, PIN, MMP, MT1-MMP, PTEN, prostate, e-cadherin

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Introduction:

Signal transducer and activator of transcription 3 (STAT3) has been implicated in many processes including development, differentiation, immune function, proliferation, survival and epithelial to mesenchymal transition (EMT) [15, 16]. In addition, constitutive activation of STAT3 has been reported in many cancers, including prostate cancer. Different studies in prostate tissues indicated that STAT3 activity was high in prostate carcinomas as compared to normal prostate tissues. Some of the studies suggest that STAT3 activation happens early and persists through prostate cancer progression and others suggest that STAT3 is activated only in prostate cancer tissues and the activation levels of STAT3 positively correlate with malignancy of tumors and increased Gleason scores [14-15-16-17-18-19-20-21-22-23]. Although these studies have led to different conclusions about STAT3 activation in early prostate cancer, they all agree that STAT3 activation is elevated in malignant tumors as compared to normal prostate tissues. In addition, analysis of the three well-characterized prostate cancer lines DU145, PC3, and LnCAP indicated that STAT3 is activated in all three of these cell lines. DU145 had the highest levels and LnCAP (the least malignant of the three) had the lowest levels of STAT3 activation. Preliminary data from our laboratory suggests that STAT3-C expression increases invasiveness in a mouse keratinocyte system. During EMT many proteases are upregulated and cell adhesion molecule expression is altered to allow for more invasive phenotypes [28-29-30-31-32]. In this proposal, we will study the role of STAT3 activation during prostate cancer progression. Collectively, the above pieces of evidence combined with the fact that STAT3 activation appears to be involved in EMT and has been linked to increased MMP-7 and MT1-MMP expression in prostate cancer cells gave rise to our hypothesis that:

STAT-3 activation regulates the expression of genes involved in EMT, including matrix metalloproteinases (MMPs), leading to increased invasive and metastatic potential in prostate cancer.

The expected results are that overexpression of STAT3-C will result in EMT, leading to increased MMP expression, and finally, resulting in increased invasiveness and metastasis *in vivo* and *in vitro*.

Body

The following progress was made in support of Task1a and b.

Task 1: Determine whether over-expression of activated STAT3 (STAT3c) in LnCap cells stimlates EMT.

Previously, we report the accomplishment of a series of assays in the LnCap cell line and the decision of switching for other prostate cancer cell line (the MDA PCa 2-b) as a more suitable line for our research. The details were described as follows and updates are including in the text:

LnCap cells were stably transfected using a CMV-STAT3c vector , [Murine Stat3 was cloned into RcCMV-Neo (InVitrogen) tagged at the 3' end with a FLAG epitope . The Stat3-C construct was made by site-directed mutagenesis (Quick-Change by Promega) using primer pairs 5' -GCTATAAGATCATGGATACCCATCCTGGTGTCTCC]. We were able to obtain five clones which were then characterized. Western blot analysis was performed on cell lysates prepared from each clone as well as the parental LNCaP line to evaluate the expression of total STAT3 protein and to confirm the presence of the Flag tag. As shown in Figure 1, although a few of the clones appeared to have an increase in expression of total Stat3 protein only one of the clones was positive for expression of the flag tag indicating successful transfection of the entire STAT3c vector. Further characterization was performed on the positive clone, hereafter referred to as LNCaP-STAT3C.

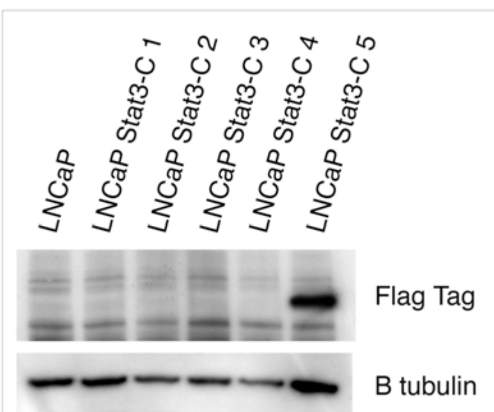


Figure 1 Western blot analysis of the parental LnCaP cell line and LnCaP -Stat3C transfected clones (from LnCaP Stat3C 1 to 5). Protein lysates were prepared from cultured cells and analysed with antibodies against (A) the Flag-tag and (B) total STAT3.

Western blot analysis was performed on cell lysates prepared from cell cultures at confluency to examine the expression of several proteins that have been shown to be critical to cell proliferation, migration and anchorage. The results of these experiments are shown in Figure 2. There were no significant alterations in the expression of Cyclin D1, VEGF, integrin α6, or MMP2 and MMP9. In addition we were unable to detect expression of MMP7 in either the LNCaP parental line or LNCaP- STAT3C. (data not shown). MMP caseinase and gelatinase activity were assessed by gel zymography. The

results are shown in Figure 3. We were unable to detect caseinase activity in either cell line (Fig. 3A) and there was no difference in gelatinase activity (MMP2, MMP9) between the two lines.

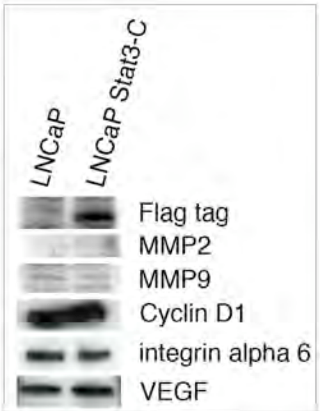
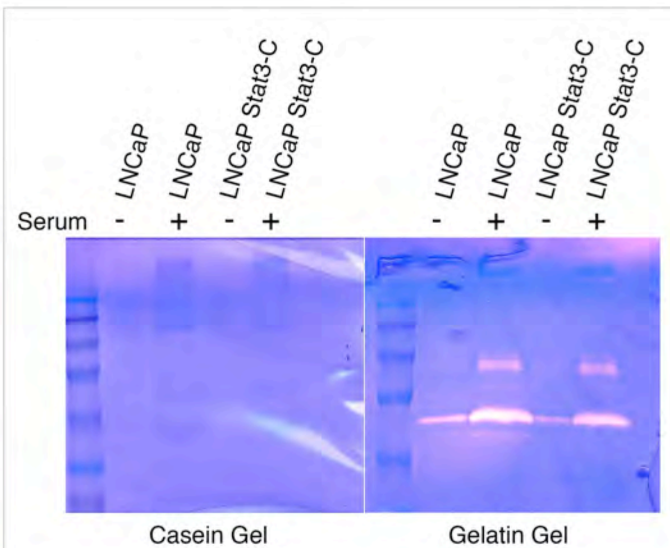


Figure 2 Analysis of protein expression by western blot using antibodies against the Flag-tag, MMP2, MMP9, Cyclin D1, Integrin α-6, and VEGF (vascular endothelial growth factor) in the LnCaP cell line and the LnCaP -Stat3C clone.



Zymography gel assay for casinase (3-A) and gelatinase (3-B) activity in both the parental LnCaP cell line and the LnCaP -Stat3C clone. Lysates were prepared from cultured cells, separated by SDS-PAGE gels containing either casein or gelatin and analysed for activity after removal of SDS.

Figure 3-a **Figure3-b**

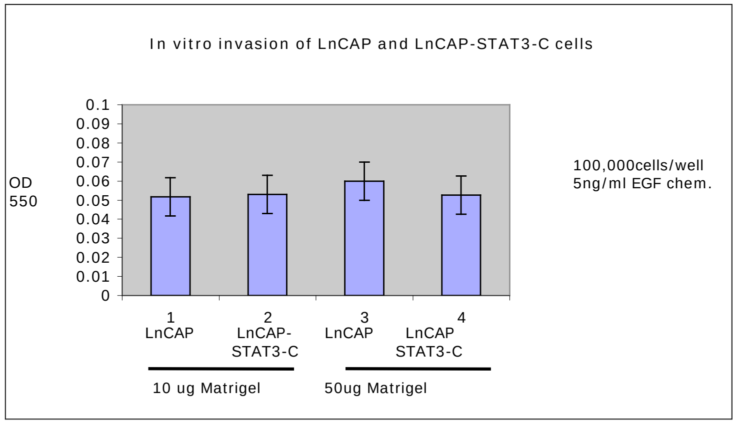
The following progress was made in support of Task 2a and b.

Task 2: Determine whether STAT3 activation increases invasiveness.

The invasive properties of the two cell lines were compared using an *in vitro* migration assay. The results are shown in Figure 4. There was no difference in migration through matrigel between the two lines. Review of the preliminary results indicated that overexpression of STAT3c did not appear to confer an invasive phenotype on the parental LNCaP cell.

Figure 4

In vitro migration assay through Matrigel membrane matrix. Assay was performed under two different Matrigel concentration (10ug and 50 ug) for both lines, LnCaP and LnCaP -Stat3C clone.



Thus, rather than progressing to part b of Task 2 which is the assessment of the invasive properties of the LNCaP-STAT3c clone in vivo, we elected to pursue an alternate strategy.

The *in vitro* experiments were repeated using a different prostate cancer cell line that was developed at MD Anderson Cancer Center, MDA PCa 2b [Nora Navone, Clinical Cancer Research Vol. 6, 1190-1197, March 2000]. The vector described above was used to transfect the cells and three clones were obtained confirmed later the presence of the gene via real time PCR (data not show). In figure 5, we show the Initial characterization of the clones by Western blot analysis revealed that all three clones were positive for the Flag-tag and had a slight increase in total STAT3 protein. In addition, phosphorylated STAT3 was slightly detected in the original MDA PC2b line and in the transfected clones. Analysis of MMPs 2, 7, and 9 revealed an increase in protein expression of MMP2 but no difference in the level of MMP9.

We were unable to detect expression of MMP7 in any of the lines (data not shown). In addition, there was an apparent decrease in the level of E-cadherin in 2 of the 3 clones (MDA Pca-STAT3c1 and 2) that correlated with increased levels of MMP2.

If, as expected, transfection of the MDA PCa cell line confers an invasive phenotype then this line will be used to examine whether this phenotype is maintained in vivo as described in Task 2a.b.

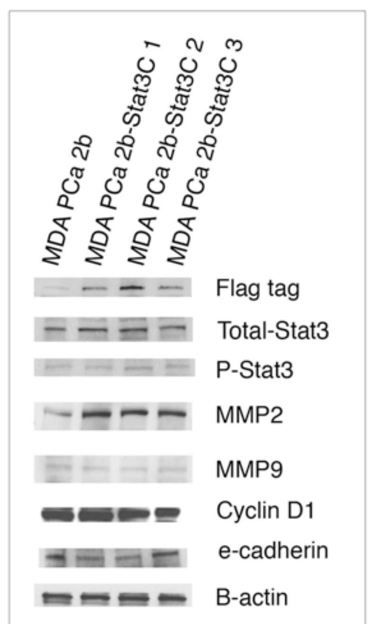


Figure 5.

Western blot analysis of MDA PCa 2b cells and Stat3C-transfected clones (MDA PCa 2b-Stat3C 1 to 3). Protein lysates were prepared from cultured cells and analyzed with antibodies against the Flag-tag, total Stat3, phospho-Stat3, MMP2, MMP9, and Cyclin D1, e-cadherin)

Immunofluoroscence was performed to check the presence of the STAT3C protein (flag-tag) in the STAT3C transfected cell lines (figure 6). e-cadherin showed lower expression in the Immunofluoroscence assay in the STAT3C transfected cell lines (figure 7). Assays were performed in all Sat3C transfected cell lines. Figures are from representative samples of the different lines.

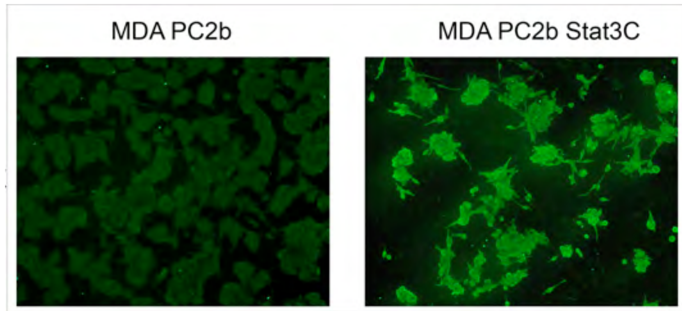


Figure 6. Immunofluoroscence in MDA PC2b cells and MDA PC2b Stat3C transfected cells confirming the presence of Stat3C (flag-tag) protein and its cytoplasmic localization.

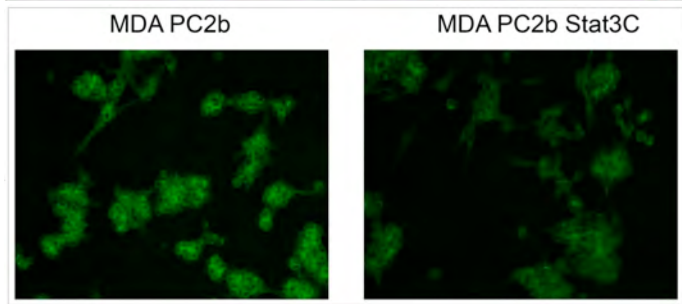


Figure 7. IF staining of e-cadherin. Transfected cells shows lower expression of e-cadherin protein.

Further analysis of the transfected cell lines revealed an increased growth rate in comparison with the parental line, while the apoptosis assays showed a lower rate in the transfected cells. (Figure 8 and 9)

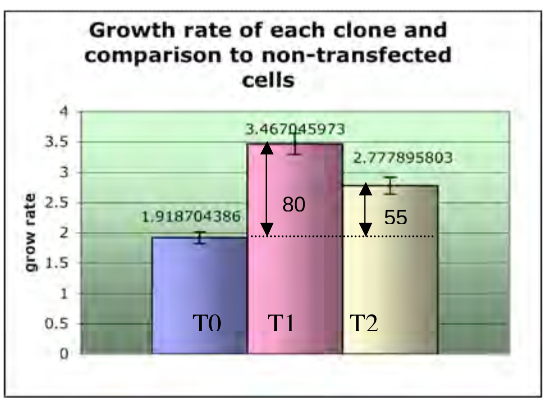


Figure 8. Proliferation assay. Parental MDA PC2b cells (T0) compared with two of the Stat3C transfected cell lines (T1, T2). Growth rate was read on the day 18th after seeding. Growth rate in the Stat3C transfected cells were 80% and 55% higher than the parental non-transfected cell line.

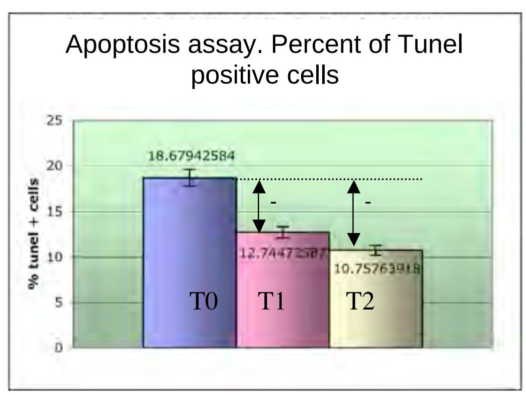


Figure 9. Apoptosis Assay. Percent of apoptotic cells was also statistically significant. 18.6 % of the cells were tunel positive in the non-transfected cells (T0), meanwhile 12.7% and 10.7% were tunel positive in the two Stat3C transfected cell lines (T1, T2).

The invasive properties of the original MDA PC2b cell line and the Stat3C transfected cell lines were compared using an *in vitro* invasion assay. The results are shown in Figure 10. There was a slight statistical significance in the difference of the invasion through matrigel between the parental line (TO) and the pool of transfected lines (TP). In order to complement the experiment, a MMPs broad spectrum inhibitor was used (GM600, Ilomastat) in replacement of the inhibitor Batimastat (BB94) originally proposed duo to the commercially unavailability of the product. Although there was a slight reduction in the number of invasive cells in the assay with the inhibitor, the difference was no significant.

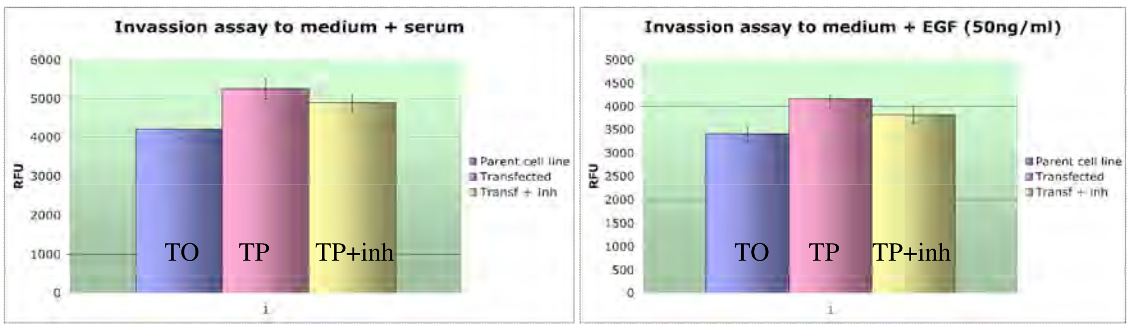


Figure 10. In vitro invasion assay through Matrigel membrane matrix. Assay was performed trough two different vehicle (medium + serum, left; and medium + EGF (50ng/ml) for both lines, MDA PC2b (T0) and MDA PC2b Stat3C pool of clones (TP). TP+inh; complementary assay with addition of MMPs inhibitor.

Review of the these results indicated that overexpression of Stat3c in the MDA PC2b prostate cancer cell lines appear to confer an increase in survival, proliferation and a mild *in vitro* invasive phenotype.

The objective of Task 2b is to determine whether expression of probasin-STAT3c in the background of BK5.IGF-1 transgenic mice leads to higher expression levels of MMPs in prostate tissues and development of more invasive tumors.

For the first part of this task (2b.a.) we had proposed to develop double transgenic mice that were hemizygous for both the BK5.IGF-1 transgene (+/-) and the Pb.Stat3c transgene (+/-). We began with six sets of breeders, each consisting of a BK5.IGF-1 transgenic male paired to a Pb.Stat3C female. Only one female has successfully given birth and each litter has been unexpectedly small. Although the breeding colony has been expanded it may be difficult to produce a sufficient number of the expected genotypes to accomplish the research goals since the male double transgenic animals represent ~ 10% of the total number of animals born.

Hence we have employed an alternate strategy. We have available in our facility PTEN knockout mice on an ICR background. It is well known that loss of one PTEN allele is a high frequency event in prostate cancer, occurring in as many as 70-80% of primary tumors (Whang PNAS 1998, Gray, Br J Cancer. 1998, Cairns Can Res 1997, Suzuki Can Res 1998) and homozygous inactivation of PTEN is associated with advanced disease and metastasis (Cantley PNAS 1999, DiChristofano Cell 2000). The tumor suppressor PTEN functions as a lipid and protein phosphatase, and is a negative regulator of signaling through the IGF-1 receptor by inhibiting the ability of PDK1 to activate AKT (Cully Nat Rev Cancer 2006, Sulis and Parsons Trends in Cell Biology 2003, Eng Hum Mut 2003). In addition it has been shown, at least in tissues other than the prostate, that there are a downstream link between mTOR and Stat3, in which mTOR is required for maximal activation of STAT3. Thus, we expected that the loss of PTEN may enhance the onset of tumor development compared with single trannsgenic and It is hoped that the additional perturbation of activated STAT via the Pb.STAT3c transgene will enhance the development and progression of prostate tumors. Breeding has begun on thelast year to produce male mice hemizygous for the Pb.STAT3c transgene and the PTEN knockout allele, and liters of the double crosses has already been scarified and the morphology, IHC analysis and statistical data are shown in the figure 13, 14, 15, 16 and table 1 respectively.

As part of Task 2b, we have been characterized the prostate of the hemizygous Pb.STAT3C male mice, aged to 6 and 12 months. Necropsy and prostate dissection was performed on 10 mice that ranged in age from 6 to 7 months. The individual lobes were harvested and snapfrozen for biochemical analysis or the genitourinary tract was removed intact and fixed in phosphate-buffered formalin.

Representive sections of the findings are shown in figure 11 and additional examples of the lesions (figure 12). To date, 16 animals were analyzed (n=8 for six months old and n=8 for 12 months old). The morphology of the anterior prostate (AP) was essentially normal and consistent with the morphology observed in nontransgenic males of the same age. The glands of the ventral prostate (VP) were predominantly normal in appearance, epithelial hyperplasia was occasionally observed and prostatic intraepithelial neoplasia (PIN) were observed in the oldest animals. Examination of the dorsalateral prostate (DP) revealed that at least one animal presented with a mix of atypical epithelial hyperplasia and cellular changes consistent with PIN.

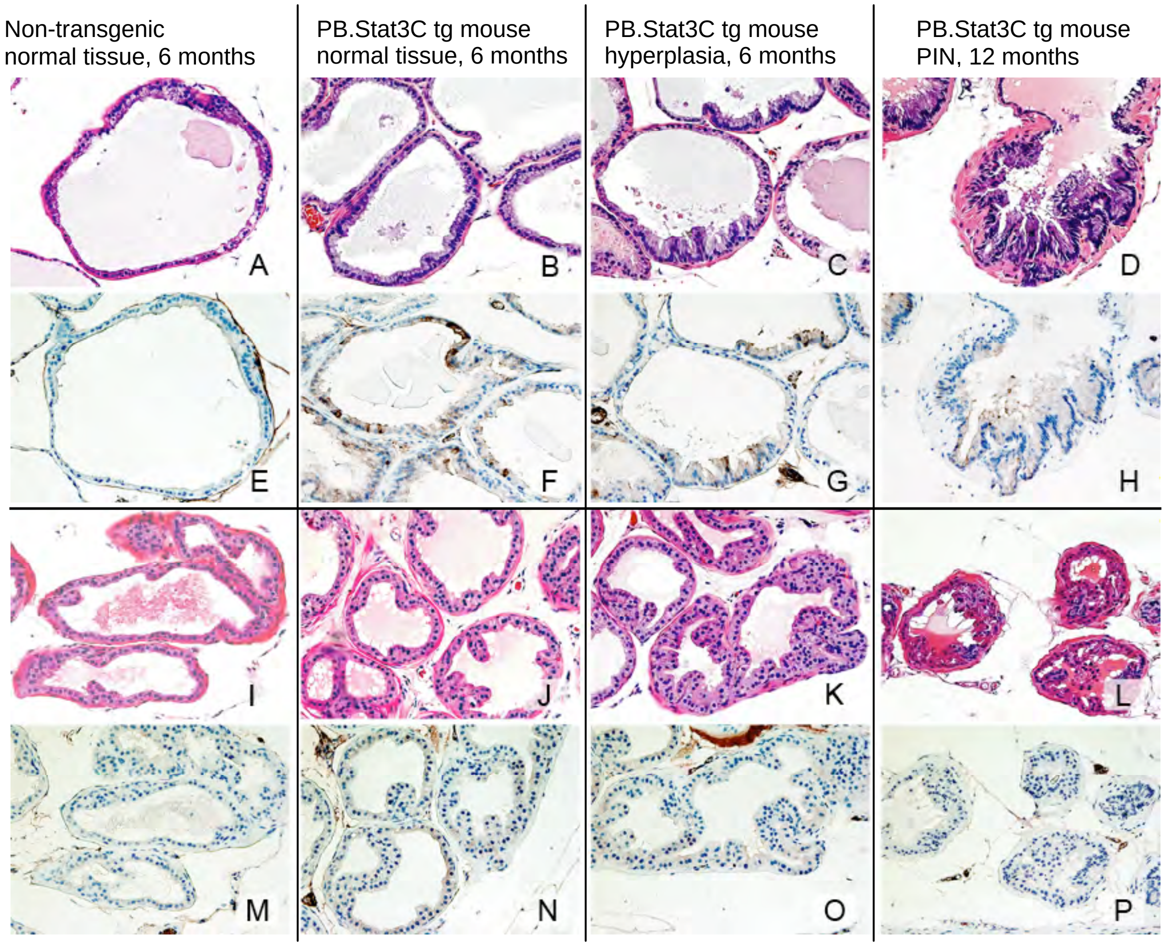


Figure 11. Representative stained sections of the Ventral (A to H) and Dorsal (I to P) Prostate from PB.Stat3C mice at both, 6 and 12 months of age. H&E staining (A-D,I-L) and IHC for the transgene (E-H,M-P) using the anti-flag antibody. The left panels (A,E,I,M) show a normal ventral prostate from a non-transgenic mouse. At 6 months of age, transgenic mice (line E) show normal tissue (B, F, J and N) and some areas of hyperplasia (C, G, K and O). At 12 months of age (D, H, L and P) lesions are more aggressive, as more pronounced epithelial tufting and also PINs are present. IHC staining for the transgene showed that it could be easily detected in normal tissue and hyperplastic glands. however, there was lower transgene expression in PIN lesions. This observation was again true for all of the PIN lesions seen in DLP of PB.Stat3C mice. It should also be noted that the level of transgene expression in DLP as assessed is consistent with the Western Blot data showing lower expression of the transgene in DLP vs. the VP. Magnification is 20X.

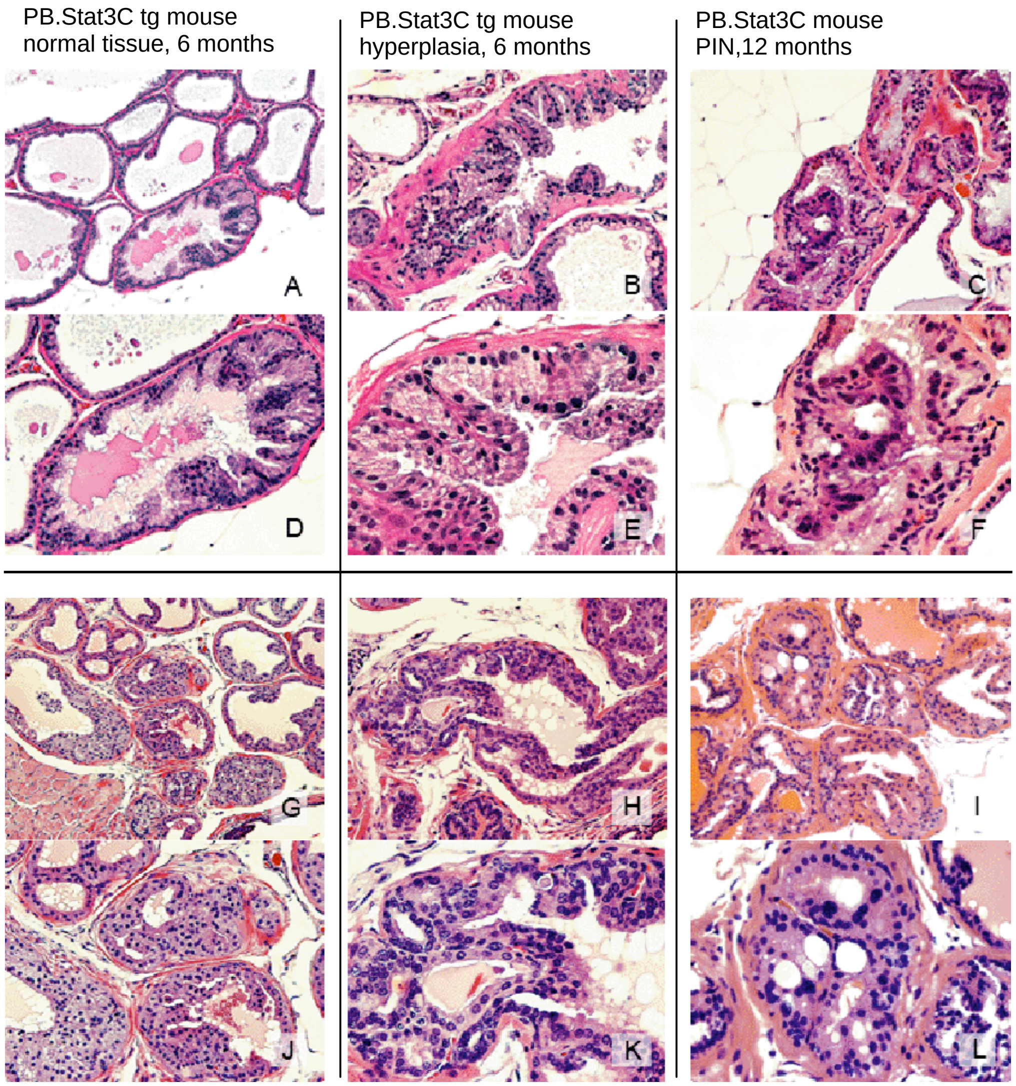


Figure 12. Additional representative lesions observed in VP (A-F) and DP (G-L) of PB.Stat3C transgenic mice. Left panels (A,D,G and J) show more examples of hyperplasia; there is an increase in the epithelial tufting, but the cells have a relatively normal appearance. The middle (B,E,H,K) and right (C,F,I,L) panels show further examples of PINs, PIN lesions were characterized by cell enlargement, karyomegaly, karyocytomegaly, nuclear atypia with apical localization and chromatin condensation and the presence of one or more prominent nucleoli. There are also some cribriform growth patterns and the formation of many small intraluminal glands. (Magnification : A and G: 10X; B, C, D, H, I and J: 20X; E, F, K and L: 40X)

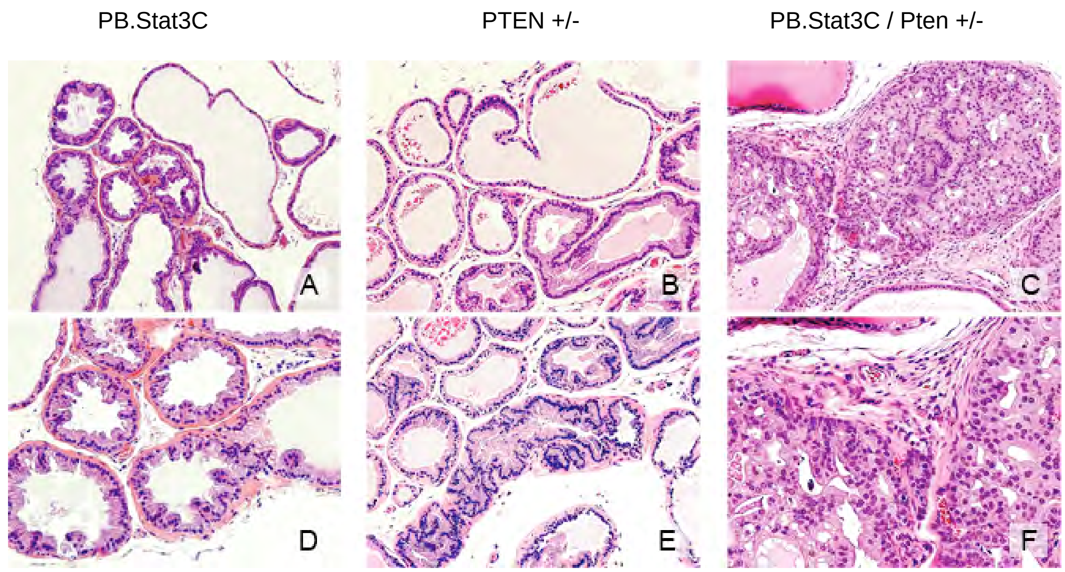


Figure 13. Pictures of representative lesions in the Ventral Prostate of the PB.Stat3C line (panels A and D), PTEN +/- mice (panels B and E) and the double transgenic mice (panels C and F) resulting from this cross. Hyperplasia and PINs developed in the PB.Stat3C and PTEN +/- lines, whereas, in the double transgenic mice, adenocarcinomas where observed in VP glands at 6 and 12 months of age.

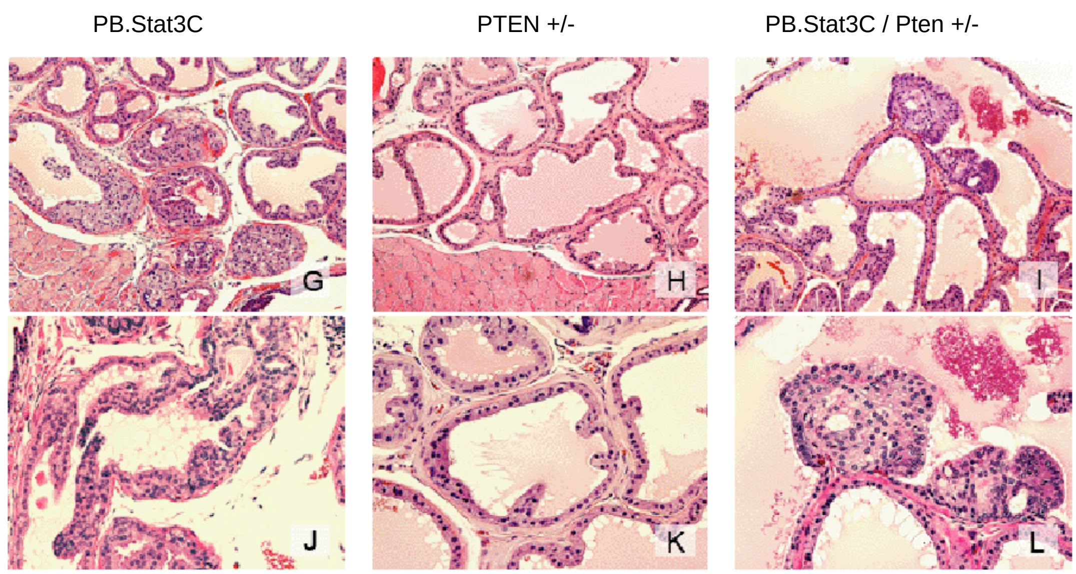
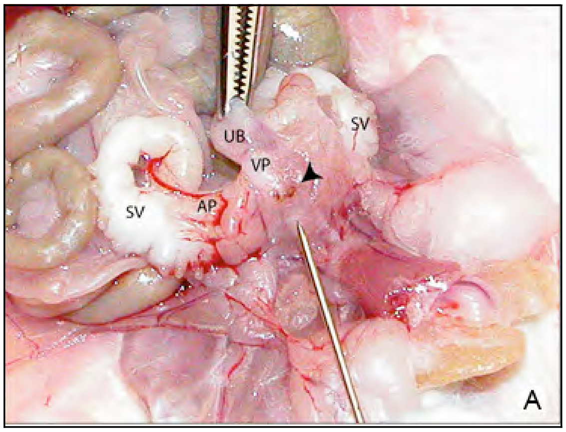
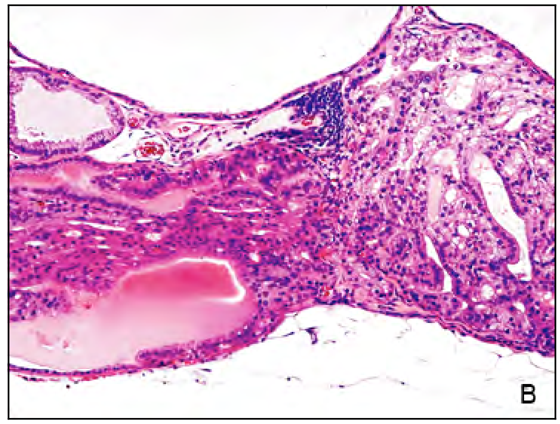


Figure 14. Pictures of representative lesions in the Dorsal Prostate of the PB.Stat3C line (G and J), PTEN mice (panels H and K) and the double transgenic mice (panels I and L) resulting from this cross. To date we have observed only hyperplasia and low grade PIN in the DP of these mice. Panels I and L show a low (10x) and higher (20x) magnification of a papillary growth in the lumen of the gland with similar features to those described in both parental lines.





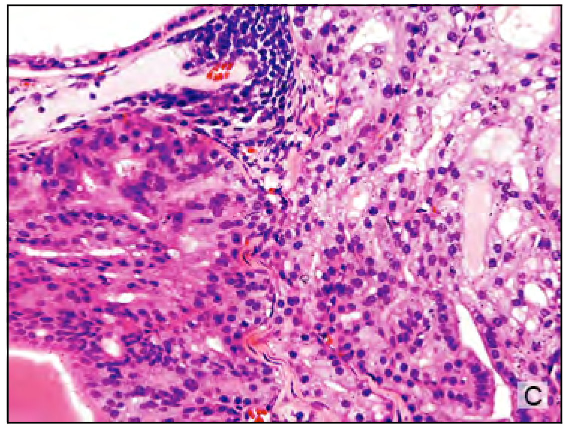


Figure 15. Examples of the lesions found in the ventral prostate of the PB.Stat3C x PTEN +/- mice . (A) Necropsy of a 12 month old PB.Stat3C x PTEN mouse showing a mass with darker coloration in the VP (arrow). (B and C) Lower and higher magnification of additional examples of adenocarcinoma in the ventral prostate of the PB.Stat3C x PTEN+/- at 12 months of age, characterized by disruption of the normal projection of the basement membrane, destructive local invasion, extension into the loose connective tissue and foci of neo-vascularization. (Magnification; B: 10x; C: 20x). Diagnosis of prostate lesions are in accordance with the Consensus Report from the Bar Harbor Meeting of the MMHCC Pathology Committee (Cancer Res., 2004).

		6 months old		12 months old	
		Ventral Prostate	Dorsal Prostate	Ventral Prostate	Dorsal Prostate
PB.Stat3C (+/-)	Hyperplasia	3/4	3/4	4/4	4/4
	PIN	0/4	1/4	3/4	2/4
	AC	0/4	0/4	0/4	0/4
PTEN (+/-)	Hyperplasia	4/4	4/4	1/1	1/1
	PIN	1/4	0/4	1/1	1/1
	AC	0/4	0/4	0/1	1/1
PB.Stat3C/ PTEN (+/-)	Hyperplasia	4/4	4/4	5/5	5/5
	PIN	2/4	2/4	5/5	5/5
	AC	1/4	0/4	3/5	0/5

Table 1. Summary of the lesions in the two parental lines (Pb.Stat3C +/- and PTEN +/-) and the crosses.

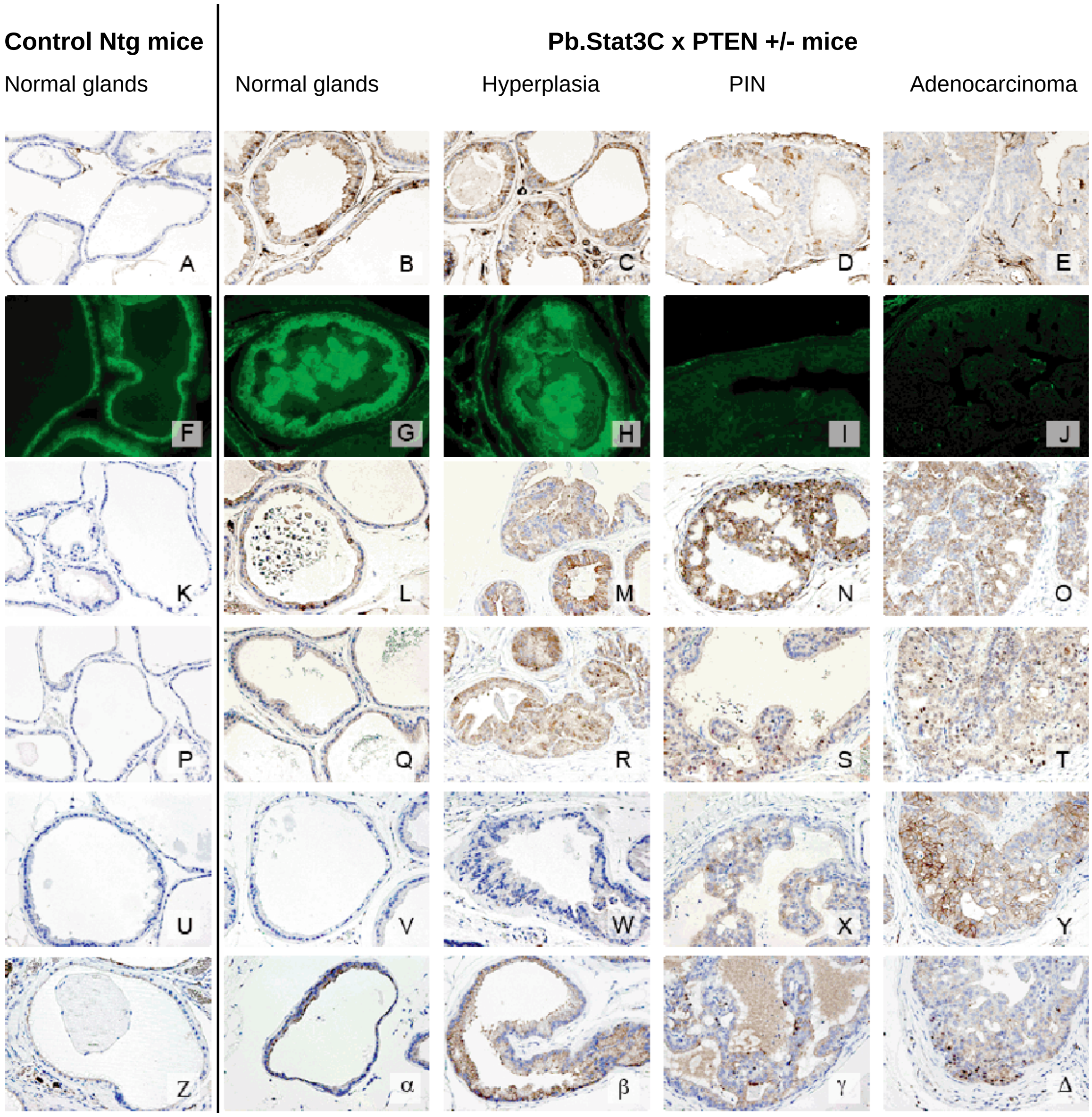


Figure 16. Immunohistochemical analyses of prostate lesions in VP of the double transgenic mice (PB.Stat3C x PTEN+/-). Stat3C transgene expression (A-E), and endogenous probasin (F-J) in the ventral prostate appear to be markedly reduced in the PIN and adenocarcinoma (D, I, E and J). P-Stat3 (P-T) , P-Akt (U-Y) and cyclin D1 (Z-D) levels progressively increased with the severity of the lesions in these mice. Note the marked nuclear staining for both P-Stat3 and cyclin D1. Despite the reduction in transgene expression, nuclear P-Stat3 levels increased with severity of the lesion. Note also that this pattern of staining was seen in all of the areas of adenocarcinoma observed in the double transgenic mouse. These preliminary analyses of double transgenic mice indicate that there are progressive changes in both Akt and Stat3 phosphorylation that may contribute to tumor progression. Further ongoing studies will confirm these findings.

Review of this section of the project in which the objective was to determine whether expression of probasin-STAT3c in the background of BK5.IGF-1 transgenic mice (change for PTEN +/- mice) leads to higher expression levels of MMPs in prostate tissues and development of more invasive tumors. Results indicate that although

PB.Stat3C transgenic mice at 12 months of age did not develop prostate tumors, they exhibited hyperplastic foci and the presentation of prostate intraepithelial neoplasias (PINs) in the ventral and dorsal lobes of the prostate at a high incidence. Similar to PB.Stat3C, the PTEN+/- mice developed hyperplasia and PIN but fully invasive tumors were not observed. However, results indicate that the overexpression of Stat3C coupled with loss of PTEN (PB.Stat3C x PTEN+/- mice) leads to a stronger phenotype, especially in the ventral prostate, including the development of adenocarcinomas as early as 6 months of age and a higher incidence at 12 months of age. The incidence of the prostate lesions, both PINs and ACs increased with age in the double transgenic mice. Additionally, preliminary IHC evaluation of adenocarcinoma in PB.Stat3C x PTEN +/- mice showed elevated pStat3, pAkt and nuclear cyclin D1. Again, Stat3C transgene expression was reduced in the more severe lesions in these mice. Non metastatic lesions were found in any parental mice they crosses.

The objective of the task 3 was to determine whether over-expression of Stat3C results in increased metastatic potential.

To test whether Stat3 activation increases the metastatic potential of prostate cancer cells, MDA PC2b cells expressing Stat3C were injected into the Ventral prostate of athymic mice (SCID) and two weeks later the pelvic and retroperitoneal lymph nodes were analyzed for metastases. In addition, other set of mice were also injected with MDA PC2b cells expressing Stat3C via IP to check invasion to the diaphragm muscle. With the use of the GM600 MMPs inhibitor (in replace of the BB94 inhibitor) we should be able to determine if the potential increase in metastatic potential is mediated though increased MMP activity.

The following table (table 2) resume the set-up for the metastasis *in vivo* experiment. Table include vehicle injected on each group of mice for the orthotopic or IP delivery and resume of the lesions found after necropsy.

Injected with	Orthotopic	Intra Peritoneal (IP)
MDA PC2b non-transfected cells ($1x10^6$ cells in 20 ul of media)	Macro: 5 possible tumors; non mice with lymphonode inflammation.	Macro: normal
	Micro: TBD	Micro: TBD
MDA PC2b Stat3C-transfected Cells ($1x10^6$ cells in 20 ul of media)	Macro: 8 possible tumors; 3 mice with lymphonode inflammation.	Macro: normal
	Micro: TBD	Micro: TBD
Control (media) (20 ul of media)	Macro: normal	Macro: normal
	Micro: TBD	Micro: TBD
Vector transfected cells ($1x10^6$ cells in 20ul)	Macro: 4 possible tumors; 1 lymphonode inflammation	Macro: normal
	Micro: TBD	Micro: TBD
MDA PC2b Stat3C transfected cells ($1x10^6$ cells in 20ul) + MMP inhibitor (GM600; 5nM)	Macro: 3 possible tumors; non lymphonode inflammation	Macro: normal
	Micro: TBD	Micro: TBD

Table 2. Metastasis assay after IP and Orthotopic delivery in SDIC mice
Ten mice were used for each experimental group (total of 100 mice)
TBD: To be determined.

Preliminary analysis of macroscopic examination after necropsy revealed an increase in the number of possible tumors after orthotopic injection of the MDA PC2b Stat3C transfected cells, 8 out of 10 and 3 lymphonode inflammation, compared with the non-transfected group, 5 out 10 and non lymphonode inflammation. Control group (medium cell-free injected) showed non tumors or lymphonode inflammation; transfected cells with empty vector, 4 possible tumors and 1 mice with lymphonode inflammation; and MDA PC2b Stat3C transfected cells + inhibitor showed 3 possible tumors and non lymphonode inflammation.

The second part of the experiment, IP injection of the different deliveries, showed no macroscopic signs of invasion to the diaphragm.

Ongoing studies and microscopic readings still remaining for this part of the experiment.

Key Research Accomplishments

***Specific Aim 1*:** Determine whether over-expression of activated Stat3 (Stat3C) in LnCAP cells stimulates EMT.

Accomplishments:

For the originally proposed cell line, LnCAP cells:

- There were no significant alterations in the expression of Cyclin D1, VEGF, integrin α6, or MMP2 and MMP9.
- In addition we were unable to detect expression of MMP7 in either the LNCaP parental line or LNCaP-Sta3C clones. MMP caseinase and gelatinase activity were assessed by gel zymography.
- We were unable to detect caseinase activity in either cell line (Fig. 3A) and there was no difference in gelatinase activity (MMP2, MMP9) between the two lines.

For the alternative proposed cell line, MDA PC2b:

- MDA PC2b Stat3C transfected cells revealed an increase in protein expression of MMP2 but no difference in the level of MMP9.
- We were unable to detect expression of MMP7 in any of the lines (data not shown).
- In addition, there was an apparent decrease in the level of E-cadherin in the clones, that correlated with increased levels of MMP2.

***Specific Aim 2a*:** Determine whether over-expression of activated STAT3 (STAT3-C) in LnCAP cells increases invasive potential *in vivo* and *in vitro*.

Accomplishments:

For the originally proposed cell line, LnCAP cells:

- There was no difference in migration through matrigel between the original LnCAP cells and the Stat3C transfected clones.
- Review of the preliminary results indicated that overexpression of STAT3c did not appear to confer an invasive phenotype on the parental LNCaP cell.

For the alternative proposed cell line, MDA PC2b:

- Review f the invasion assays indicated that overexpression of Stat3c in the MDA PC2b prostate cancer cell lines appear to confer an increase in survival, proliferation and a mild *in vitro* invasive phenotype.

***Specific Aim 2b*:** Determine whether expression of PB-STAT3-C in the background of BK5-IGF-1 (switching for PTEN +/- as alternative strategy) transgenic mice leads to higher expression levels of MMPs in prostate tissues and development of more invasive tumors (or have earlier onset of invasion).

Accomplishments:

- Results indicate that although PB.Stat3C transgenic mice at 12 months of age did not develop prostate tumors, they exhibited hyperplastic foci and the presentation of prostate intraepithelial neoplasias (PINs) in the ventral and dorsal lobes of the prostate at a high incidence.
- Similar to PB.Stat3C, the PTEN+/- mice developed hyperplasia and PIN but fully invasive tumors were not observed. However, results indicate that the overexpression of Stat3C coupled with loss of PTEN (PB.Stat3C x PTEN+/- mice) leads to a stronger phenotype, especially in the ventral prostate, including the development of adenocarcinomas (AC) with stromal invasion, as early as 6 months of age and a higher incidence at 12 months of age.
- The incidence of the prostate lesions, both PINs and ACs increased with age in the double transgenic mice. Additionally, preliminary IHC evaluation of adenocarcinoma in PB.Stat3C x PTEN +/- mice showed elevated pStat3, p-Akt and nuclear cyclin D1. Again, Stat3C transgene expression was reduced in the more severe lesions in these mice.

Specific Aim 3: Determine whether over-expression of STAT3-C results in increased metastatic potential.

Acomplishments:

- Macroscopic examination of the prostate and lymphonode of the SDIC mice showed an increase number of possible tumors when injected with MDA PC2b Stat3C transfected cells. Microscopic examination for the confirmation of the tumors as well as the possible metastatic infiltration of the local lymphonodes still remaining.
- For the morphologic characterization of the Pb.Stat3C mice or the insertion of the gene in the PTEN background, although the crosses did not show metastatic tumors to lymphonodes or others tissues, they did showe the development of ACs with local invasion.

Reportable Outcomes

The following abstracts have resulted from the present founded investigation and were presented for an oral presentation in the AACR annual meting held in Los Angeles, april 2007 and a poster presentation in the ImPACT meeting held in Atlanta, Georgia, august 2007 respectively.

Stat3 Activation and Prostate Cancer Progression.

Jorge Blando; Manolis Demetrieou; Steve Carbajal; Linda Beltran; John DiGiovanni.
Department of Carcinogenesis, The University of Texas, M.D. Anderson Cancer Center, Science Park-Research Division, Smithville, Texas 78957.

Signal transducer and activator of transcription 3 (STAT3) has been implicated in many processes including development, differentiation, immune function, proliferation, survival and epithelial to mesenchymal transition (EMT). Constitutive activation of STAT3 has been reported in many cancers, including prostate cancer, suggesting an important role for STAT3 in tumorigenesis. Despite consistent reports of heightened STAT3 activation in prostate carcinomas, as compared to normal prostate tissue, the literature examining STAT3 activation in early prostate cancer remains inconclusive. Several studies suggest STAT3 activation in prostate tissue occurs early and persists throughout prostate cancer progression, while others report STAT3 activation occurs only in prostate cancer tissue, positively correlating with tumor malignancy and Gleason score. During EMT, protease upregulation, such as matrix metalloproteinases (MMPs), and altered cell adhesion molecule expression, specifically e-cadherin, allows for a more invasive phenotype. To examine the effect of Stat3 activation on prostate cancer progression, transgenic mice were generated to express a constitutively active form of Stat3 (STAT3C) using the probasin (PB) promoter (PB.STAT3C transgenic mice). Transgenic and non-transgenic mice were sacrificed at six months of age and prostate tissue was collected for histopathological analysis. Hyperplastic changes were observed in both transgenic and non-transgenic mice in the ventral and dorsal prostate, while neoplastic alterations, such as prostate intraepithelial neoplasia (PIN), were only observed in STAT3C transgenic prostates. Currently, we are analyzing additional PB.STAT3C mice and breeding the PB.STAT3C mice together with PTEN null, PB.PKC epsilon, and K5.IGF-1 mice. An *in vitro* study was performed to further examine the involvement of Stat3C in prostate cancer progression. MDA PCa 2b prostate cells were transfected to overexpress STAT3C protein. Western blot analysis revealed an increased expression of matrix metalloproteinase 2 (MMP2) in STAT3C overexpressing cells, as compared to the control, while e-cadherin expression was decreased in the transfected clones, confirming the regulation of EMT associated proteins by STAT3. Currently, the *in vitro* data suggest a role for STAT3 activation in prostate cancer progression through differential regulation of EMT associated proteins. Ongoing studies in the PB.STATC transgenic mice will provide further evidence for or against this hypothesis.

Stat3 Activation and Prostate Cancer Progression.

Development and characterization of transgenic mice expressing Constitutively active stat3 (stat3c) in prostate.

Jorge Mario Blando; Manolis Demetriou; Steve Carbajal; Linda Beltran; John DiGiovanni.
Department of Carcinogenesis, The University of Texas, M.D. Anderson Cancer Center, Science Park-Research Division, Smithville, Texas 78957.

Signal transducer and activator of transcription 3 (STAT3) has been implicated in many processes including development, differentiation, immune function, proliferation, survival and epithelial to mesenchymal transition (EMT). Constitutive activation of STAT3 has been reported in many cancers, including prostate cancer, suggesting an important role for STAT3 in tumorigenesis. Despite consistent reports of heightened STAT3 activation in prostate carcinomas, the literature examining STAT3 activation in early prostate cancer remains inconclusive. Several studies suggest STAT3 activation in prostate tissue occurs early and persists throughout prostate cancer progression, while others report STAT3 activation occurs only in prostate cancer tissue, positively correlating with tumor malignancy and Gleason score. During EMT, protease upregulation, such as matrix metalloproteinases (MMPs), and altered cell adhesion molecule expression, specifically e-cadherin, confers a more invasive phenotype. To examine the effect of Stat3 activation on prostate cancer progression, transgenic mice were generated that express a constitutively active form of Stat3 (STAT3C) using the probasin (PB) promoter (PB.STAT3C). Transgenic and nontransgenic mice were sacrificed at six months of age and prostate tissue was collected for histopathological analysis. Hyperplastic changes were observed in prostate of both transgenic and nontransgenic mice although neoplastic alterations, such as low grade prostate intraepithelial neoplasia (PIN), were only observed in transgenic mice. In addition, the PB.STAT3C mice were crossed to PTEN heterozygous null (PTEN$^{+/-}$) mice as well as PB.PKCε and BK5.IGF-1 transgenic mice. Six-month-old mice were sacrificed and histological analysis showed an increase in both the incidence and severity of the lesions in the prostate of the PB.STAT3C mice coupled with loss of PTEN (PTEN$^{+/-}$/PB.STAT3C). In this regard, hyperplasia and high grade PIN were observed in the prostate of the PTEN$^{+/-}$/PB.STAT3C mice versus hyperplasia and low grade PIN only in PB.STAT3C mice of the same age. In contrast, PB.PKCepsilon/PB.STAT3C mice had a weaker pathologic phenotype, displaying only moderate hyperplasia. BK5.IGF/STAT3C crosses are still in progress. An *in vitro* study was performed to further examine the involvement of STAT3C in prostate cancer progression. MDA PCa 2b prostate cells were transfected to overexpress STAT3C protein. Western blot analysis revealed an increased expression of MMP2 in STAT3C overexpressing cells, as compared to the control. To date, the *in vitro* data suggest a role for STAT3 activation during prostate cancer progression through differential regulation of EMT associated proteins, as well as by altering signaling pathways involved in cell survival and proliferation.
IMPACT: The combined use of human prostate cancer cell lines and mouse prostate cancer models should further provide data most closely translatable to the patient situation. The understanding of the role of STAT3 activation and the identification of downstream molecular targets may have its greatest impact in the development of novel therapies for the treatment of invasive and metastatic prostate cancer.

Conclusion

Although PB.Stat3C transgenic mice at 12 months of age did not develop prostate tumors, they exhibited hyperplastic foci and the presentation of prostate intraepithelial neoplasias (PINs) in the ventral and dorsal lobes of the prostate at a high incidence. Similar to PB.Stat3C, the PTEN+/- mice developed hyperplasia and PIN but fully invasive tumors were not observed. However, preliminary results indicate that the overexpression of Stat3C coupled with loss of PTEN (PB.Stat3C x PTEN+/- mice) leads to a stronger phenotype, especially in the ventral prostate, including the development of Adenocarcinomas as early as 6 months of age and a higher incidence at 12 months of age. The incidence of the prostate lesions, both PINs and ACs increased with age in the double transgenic mice.

MDA PC2b cells expressing Stat3C gene revealed an increase in protein expression of MMP2. In addition, there was an evident decrease in the level of E-cadherin in the transfected clones. To date, the *in vitro* data suggest a role for STAT3 activation during prostate cancer progression through differential regulation of EMT associated proteins, as well as by altering signaling pathways involved in cell survival and proliferation. In addition, the expression constitutively active Stat3 in MDA PC2b prostate cancer cell lines confer a mild *in vitro* invasive phenotype.

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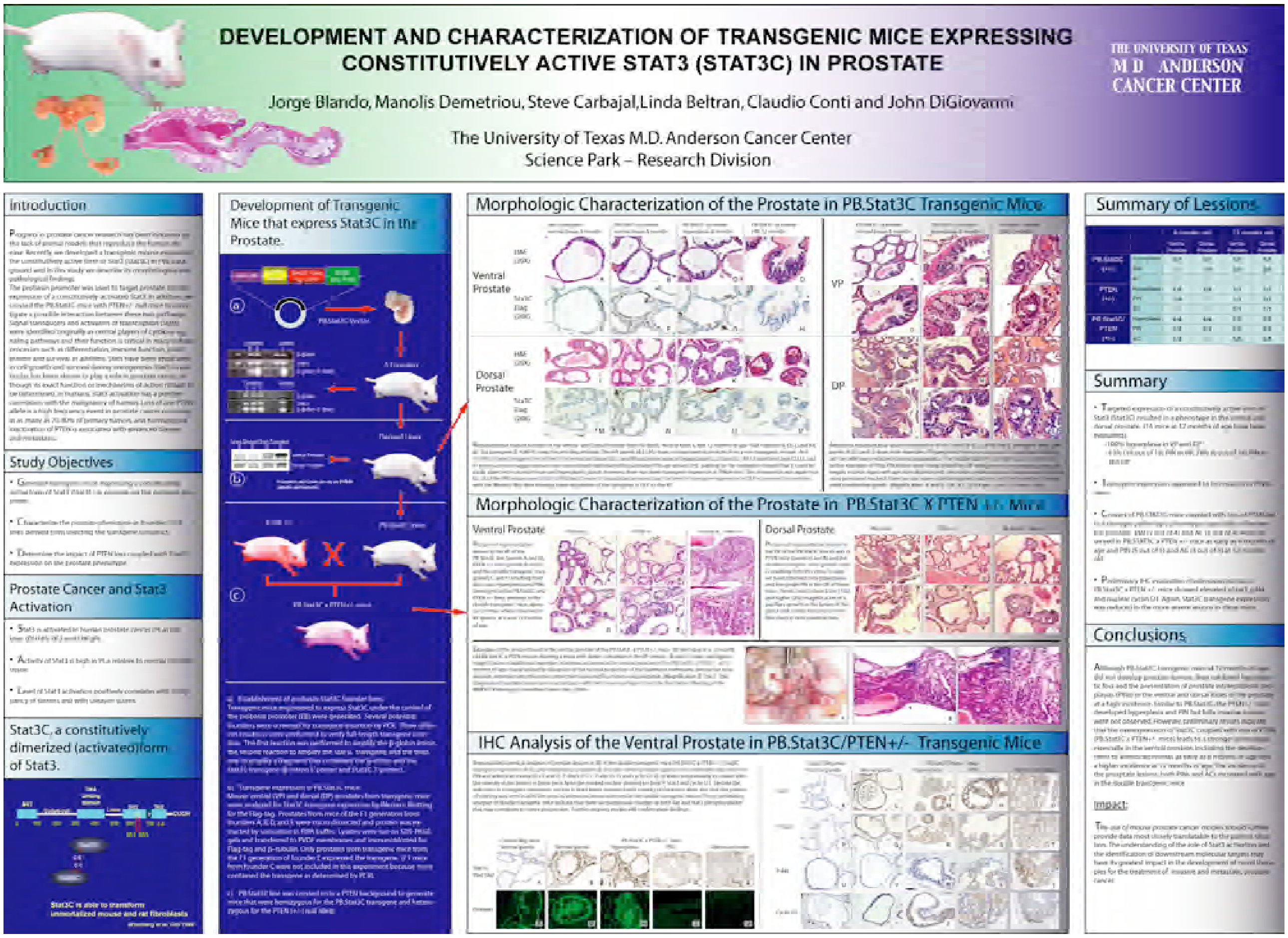
Appendices

Abstract for the AACR annual meting held in Los Angeles, april 2007.

#4938 Stat3 activation and prostate cancer progression. Jorge M. Blando, Manolis Demetrieou, Steve Carbajal, Linda Beltran, John Digiovanni. *UT M.D. Anderson Cancer Ctr., Smithville, TX.*

Signal transducer and activator of transcription 3 (STAT3) has been implicated in many processes including development, differentiation, immune function, proliferation, survival and epithelial to mesenchymal transition (EMT). Constitutive activation of STAT3 has been reported in many cancers, including prostate cancer, suggesting an important role for STAT3 in tumorigenesis. Despite consistent reports of heightened STAT3 activation in prostate carcinomas, as compared to normal prostate tissue, the literature examining STAT3 activation in early prostate cancer remains inconclusive. Several studies suggest STAT3 activation in prostate tissue occurs early and persists throughout prostate cancer progression, while others report STAT3 activation occurs only in prostate cancer tissue, positively correlating with tumor malignancy and Gleason score. During EMT, protease upregulation, such as matrix metalloproteinases (MMPs), and altered cell adhesion molecule expression, specifically e-cadherin, allows for a more invasive phenotype. To examine the effect of Stat3 activation on prostate cancer progression, transgenic mice were generated to express a constitutively active form of Stat3 (STAT3C) using the probasin (PB) promoter (PB.STAT3C transgenic mice). Transgenic and non-transgenic mice were sacrificed at six months of age and prostate tissue was collected for histopathological analysis. Hyperplastic changes were observed in both transgenic and non-transgenic mice in the ventral and dorsal prostate, while neoplastic alterations, such as prostate intraepithelial neoplasia (PIN), were only observed in STAT3C transgenic prostates. Currently, we are analyzing additional PB.STAT3C mice and breeding the PB.STAT3C mice together with PTEN null, PB.PKC epsilon, and K5.IGF-1 mice. An *in vitro* study was performed to further examine the involvement of Stat3C in prostate cancer progression. MDA PCa 2b prostate cells were transfected to overexpress STAT3C protein. Western blot analysis revealed an increased expression of matrix metalloproteinase 2 (MMP2) in STAT3C overexpressing cells, as compared to the control, while e-cadherin expression was decreased in the transfected clones, confirming the regulation of EMT associated proteins by STAT3. Currently, the *in vitro* data suggest a role for STAT3 activation in prostate cancer progression through differential regulation of EMT associated proteins. Ongoing studies in the PB.STATC transgenic mice will provide further evidence for or against this hypothesis.

Abstract for a poster presentation in the ImPACT meeting held in Atlanta, Georgia, august 2007.



Curriculum Vitae

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Education:

2002 — **Doctor of Veterinary Medicine**.
National University of La Pampa, School of Veterinary Medicine, General Pico, La Pampa, Argentina

Membership: **Member of the American Association for Cancer Research**

Languages:
Spanish (mother tongue)
English (second language)

Research and Professional Experience:

2004-present	**Research Investigator, Post Doc** Department of Carcinogenesis The University of Texas M.D. Anderson Cancer Center Science Park-Research Division Task and Responsibilities: Study of "Prostate Carcinogenesis and Stat 3 activation during Prostate Cancer Progression in Transgenic Mice" Study of "PTEN Deficiency and it penetrancy for Invasive Prostate Carcinoma in C57Bl/6 Mice" Study of "Skin Carcinogenesis and Susceptibility to DMBA-TPA in Transgenic Mice" Study of "Photocarcinogenesis and Suceptibility to UV radiation in Transgenic Mice"
2002-2004	**Veterinary Clinician.** Clearlake Veterinary Clinic, 3224 Emerson St. Clearlake, CA, 95422.
2001-2002	**Coordinator of the Pathology, Histology and Image Services.** School of Veterinary Medicine, National University of La Pampa, Argentina.
2001	**Research Assistant.** Task and responsibilities: Study of "*Staphylococus aureus* as the Cause of Mastitis in Bovines of the Province of La Pampa, Argetina" Department of Microbiology and Immunology, School of Veterinary Medicine, National University of La Pampa, Argentina.
1999-2000	**Research Assistant.** Task and responsibilities: Study of Characteristics of Non-tuberculoses *Mycobacterium* on Lands of Province of La Pampa, Argentina. Department of Microbiology and Immunology, School of Veterinary Medicine, National University of La Pampa, Argentina.

Skill and duties, (but not limited to):

- Clinical experience in small animals including dogs, cats and rodents;
- Animal husbandry of GEM, i.e., design breeding schemes based on patterns of inheritance and phenotype;
- Experience in experimental design;
- Surgical and microsurgical procedures (surgical expertise, perioperative care, anesthesia and analgesia);
- Generation of orthotopic tumors;
- Provides technical advice to multiple investigators and collaborators on utilizing animal models for cancer related research and in the performance of animal-based experimental techniques, which include providing surgical expertise, perioperative care, anesthesia and analgesia, and approved euthanasia methods.
- Gross and histologic diagnosis and description of the pathology of the prostate, skin, thymus and other tissues in rodents;
- Image Diagnosis; X-ray and Ultrasound procedures;
- Experience in the use of digital imaging systems, digital cameras, confocal microscopes, etc.
- Interpretation and analysis of laboratory data;
- Experience in molecular pathology;
- Experience in comparative pathology, which involves the investigation and comparison of disease in various animals and humans;
- Cell assays, cell line transfections, *in vitro* and *in vivo* invasion assays, metastasis assays;
- Protein and Molecular techniques and procedures; including genotyping, western blot analysis, Zymography, immunohistochemistry, immunofluoresence , ELISA, etc.
- RNA, DNA and protein isolation from various sample sources.
- Knowledge of the cellular and molecular biology, cancer biology and cellular signaling pathways;
- Knowledge of molecular mechanisms involved in the development and progression of cancer;

- Discovery of novel prognostic molecular targets for potential therapeutic applications in the treatment of prostate cancer;
- Scientific analysis and published reports of the subject of study in manuscript form in peer reviewed journals and presentation of data at scientific meetings;
- Strong computer skills in PC and MAC; Word, Excel, Power Point, File maker pro, Image and Photo editors, Image Diagnosis (Imago Pro plus), etc.

Abstracts, Publications, Oral presentations and Communication to Congresses:

"Development and characterization of transgenic mice expressing Constitutively active stat3 (stat3c) in prostate"
Jorge Blando, Manolis Demetriou, Steve Carbajal, Linda Beltran, Claudio Conti, John Digiovanni.
Meeting of Innovative Minds in Prostate Cancer Today (IMPaCT), Atlanta, Georgia, hosted by the US Department of Defense Prostate Cancer Research Program. September 2007. Poster Presentation.

"Stat3 Activation and Prostate Cancer Progression"
Jorge Blando, Manolis Demetriou, Steve Carbajal, Linda Beltran, John DiGiovanni.
Annual meeting for the American Association for Cancer Research, Los Angeles, California, April 2007. Oral Presentation.

"PTEN Deficiency is Fully Penetrant for Invasive Prostate Carcinoma in C57Bl/6 Mice"
Jorge Blando, Melisa Portis, Fernando Benavides, Jeri Kim, Gordon Mills , Claudio Conti, and Cheryl Lyn Walker.
Keystone Symposia. PI 3-Kinase Signaling Pathways in Disease. February 15-20, 2007. Santa Fe, New Mexico. Poster presentation.

"Enhanced skin carcinogenesis in transgenic mice expressing the human cyclin D1b (CCND1b) variant".
Paola Rojas[1], Fernando Benavides[2], Marcelo L. Rodriguez-Puebla[3], **Jorge Blando[2]**, Carlos Perez[1], Erik S. Knudsen[4], David G. Johnson[1], Adrian M. Senderowicz[5], Claudio J. Conti[1]
American Association for Cancer Research, Los Angeles, California, April 2007.

“Paradoxical Protective Role of Cathepsin L in Mouse Skin Carcinogenesis”.
Benavides F, Perez CJ, **Blando J**, Contreras O, DiGiovanni J, Conti CJ. VII International Skin Carcinogenesis Conference. Austin, TX, November 2006.

“Characterization and High Resolution Mapping of *LUCA*, a new Mouse Hair Loss Mutation”
Perez CJ; Mecklenburg L; Mirabzadeh E; Aubin I; **Blando J**; Contreras O; Guénet J-L; DiGiovanni J; Conti CJ; Benavides F
XX International Mouse Genome Conference IMGC 20th, Charleston, SC, US.
November, 12-16, 2006.

“The Radiation-Induce *nakt (nkt)* Allele Is a Loss-of-Function Mutation of the Mouse Cathepsine L”
Fernando Benavides; Carlos Perez; **Jorge Blando**; Jean-Louis Guenet; and Claudio Conti.
The Journal of Immunology
University of Texas M. D. Anderson Cancer Center Science Park-Research Division
Smithville, TX 78957.
Jan, 15, 2006.

“Inoculation Techniques in Animals of Laboratory”

Blando, J.M.; Staskevich, A; Oriani, S.D.
II Workshop of Science and Techniques
School of Veterinary Medicine, National University of La Pampa.
December 2001.

“Characteristics of Nontuberculous *Mycobacterium* isolates from soil of Province of La Pampa, Argentina”
Oriani, D.S.; **Blando, J.M.**; Garcia Montero, C; Rodriguez Gomez, J; Sagardoy, MA.
XIII Scientific Meeting Argentine Association of Veterinarians of Diagnostic Laboratories, Merlo, San Luis.
November 2000.

Published Book:

"Lecciones de Bacteriologia y Micologia"
(Lessons in Bacteriology and Mycology)
Oriani DS; **Blando, J.M.**; Diab, SS; Gazia, JA.
School of Veterinary Medicine, National
University of La Pampa, march 2002.
ISBN 950-863-0477.

Papers in preparation:

"PTEN Deficiency is Fully Penetrant for Invasive Prostate Carcinoma in C57Bl/6 Mice"
Jorge Blando, Melisa Portis, Fernando Benavides, Jeri Kim, Gordon Mills , Claudio Conti, and Cheryl Lyn Walker.

"Activated Akt Pathway Results in Angiogenesis, Cell proliferation, Loss of cell to cell adhesion and the Interaction between Stabilized β-Catenin and Androgen Receptor in IGF-1 induced Prostate Carcinogenesis"
Jorge Blando, Shin-ichiro Maruya, Takashi Matsumoto, Linda Beltrán, Laura Elizondo, Kaoru Kiguchi, and John DiGiovanni.

"Enhanced skin carcinogenesis in transgenic mice expressing the human cyclin D1b (*CCND1b*) variant"
Paola Rojas, Fernando Benavides, Marcelo L. Rodriguez-Puebla, **Jorge Blando**, Carlos Perez, Erik S. Knudsen, David G. Johnson, Adrian M. Senderowicz, Claudio J. Conti

"Mice overexpressing PKC epsilon in the prostate show decreased apoptosis and increased proliferation after castration"
Benavides F, **Blando J**, Kazanietz M.

"Paradoxical Protective Role of Cathepsin L in Mouse Skin Carcinogenesis"
Benavides F, Perez CJ, **Blando J**, Contreras O, DiGiovanni J, Conti CJ.

Awards:

Postdoctoral Traineeship Award from the Department of Defense.
Proposal Title: "The role of Stat3 Activation During Prostate Cancer Progression"
M.D. Anderson Cancer Center

Science Park-Research Division
Texas, USA. August 2005.

First Prize for the best Seminar in the Conference of Science and Technology.
"Environmental Mycobacteria on Lands of Province of La Pampa and its Possible Interference with the Diagnosis of Tuberculosis by Intradermo-Reaction in Bovines."
Sagardoy, M.; Oriani, D.S.; Toso, R.; Alvarez Rubianes, N.; Bruni, M.; Filipino, S.; Cachou, P.; **Blando, J.M**.
School of Veterinary Medicine, National University of La Pampa, Argentine.
December 1999.

Fourth best GPA (4th out of 50)
School of Veterinary Medicine, National University of La Pampa, Argentine.
December 1999.

<u>Manuscripts reviewed for the Molecular Carcinogenesis journal:</u>

FGF-1-induced matrix metalloproteinase-9 expression in breast cancer cells is mediated by increased activity of NF-kB and activating protein-1
Gina Lungu ,Lina Covaleda,Odete Mendes, Heidi Martini-Stoica1 George Stoica.

Caveolin-1 upregulates glycosylation of CD147 on murine Hepatocarcinoma cell, Matrix Metalloproteinase-11 expression and cell Invasion ability *in vitro*
Li Jia, Shujing Wang, Huimin Zhou, Jun Cao, Wei Wei, Jianing Zhang.

<u>Certificates and Courses:</u>

Safety in the Laboratory and Clinical Environment.
The University of Texas; MD Anderson Cancer Center, May 4, 2004.

Sedation 1-2-3, Getting Comfortable with Alpha-2 Agonists.
Mendocino Lake Veterinary Medical Association.
April 16, 2003.

Inoculation Technique on animals of Experimentation.
School of Veterinary Medicine, National University of La Pampa.
December 13, 2001

Respiratory and Digestive Diseases in Cats.
School of Veterinary Medicine, National University of La Pampa.
October 17, 2001.

Zoonoses Seminar.
School of Veterinary Medicine, National University of La Pampa.
October 6, 2000.

Methodology of Scientific Investigation.
School of Veterinary Medicine, National University of La Pampa.
September 8, 2000.

Seminar in Improvement of Food Quality.
School of Veterinary Medicine, National University of La Pampa.
May 19, 2000.

Gross and Microscopic Pathological Techniques for Diagnosis of Ovine Pathologies caused by Metacestodes and its Differential Diagnostics.
School of Veterinary Medicine, National University of La Pampa.
May 8, 2000.

Fourth Argentine Seminar of Veterinary Surgery; Fourth Colloquium of Teaching of Surgery.
School of Veterinary Medicine, National University of La Pampa.
October 5, 1996.

Histological and Cytological Techniques.
School of Veterinary Medicine, National University of La Pampa.
August 28, 1996.